Biennial Review Form

SOP Title: Operating Procedures for Holocellulose and ∂-Cellulose

Originating Branch, or project, or group and number: WEB SIP/AP.01 **Version**: 2.00

(e.g., RCB 2.0, TERA 1.1, WRS 14A)

Original Preparer: Jillian Gregg, Modified by Renee Brooks

QA certification date of this version: February 13, 2002

BIENNIAL REVIEWS [a]

Date	EPA Reviewer (signature/title: PI, or project leader, or WACOR w/ WA#)
9/14/05	L. Renee Brooks

[a] **Signature documents the biennial review when no revisions are deemed necessary.**

If a modified version of this SOP is being followed these revisions should be submitted for review and approval.

Operating Procedures for Holocellulose and α-Cellulose Extraction

A. Signature Page

Sip/AP.01

Version 2.00

January 14, 2002
Modified May 13, 2003

Modified by:

J. Renee Brooks Date: 8/3/05

J. Renee Brooks, ISIRF Director

Approval:

Date: 8/4/05

William Hogsett, WEB Branch Chief

B. Table of Contents

C. Introduction

Holocellulose and α-cellulose extraction procedures are available in WED's stable isotope research facility (ISIRF). This procedure extracts the holocellulose from dried plant tissues, and is useful for determining the isotopic signatures of structural carbon ($\delta^{13}C$) and α-cellulose for oxygen ($\delta^{18}O$) formed at the time of tissue development. Advantages of using holocellulose or α-cellulose for $\delta^{13}C$ and $\delta^{18}O$ analyses include the lack of variation in compound composition (*i.e.* the amount of lignin versus starch), or influence of compounds formed after development is complete. Analysis of δD/H signatures require an extra step of cellulose nitration where the exchangeable H atoms of the hydroxyl groups are replaced with nitrate so subsequent analyses will include only the structurally bound H laid down in the cellulose at the time of formation. This step will not be performed in ISIRF, but both procedures can be reviewed in more depth in Leavitt and Danzer (1993). The details for α-cellulose for oxygen ($\delta^{18}O$) are given in Sternberg (1989).

Overall, holocellulose extraction involves four steps:

1) Placement of dried ground tissue in extraction bags,
2) Extraction of waxes, oils and resins in the soxhlet apparatus,
3) Boiling to remove hydrophilic compounds, and
4) Bleaching of the inorganic salts and low molecular weight polysaccharides (including gums & starches).

Further extraction to α-cellulose for oxygen ($\delta^{18}O$) will include soaking in NaOH solution, then in acetic acid to remove holocellulose

Investigators interested in extracting holocellulose or α-cellulose will perform the procedures outlined in this SOP. The corresponding Safety Plan is Archive #1 in the ISIRF QA book. The total time necessary to extract 150 samples is 2 weeks. However, batches can be overlapped so the soxhlet extraction and bleaching steps occur simultaneously.

D. Objective Statement

The objective of this Sip/AP is to provide instructions on how to obtain holocellulose or α-cellulose from plant tissues from the extraction equipment in WED's Integrated Stable Isotope Research Facility.

E. Equipment, Glassware, and Chemicals needed

Equipment:

- ANKOM** filter bag, pore size 50 (+/- 15) microns, made from nitrogen free polyester
- ANKOM heat sealer
- heating mantle
- heated stir-plate
- stir bars
- ring stand and clamps
- electronic pH meter
- conductivity meter
- drying oven
- heat resistant gloves
- viton gloves

Glassware:

- soxhlet extractor

- 500 ml round bottom flasks
- 1000 ml beaker
- 500 ml erlenmeyer flask

Chemicals:

- toluene, 200 ml per run
- ethanol (100 %), 400 ml per run
- sodium chlorite ($NaClO_2$, tech. grade), ~30 g per run
- glacial acetic acid (100 %), ~110 ml per run
- (deionized water, > 1000 ml per run)
- NaOH, ~170 g per run

**Ankom Technology Corp.
2052 O'Neil Road
Macedon, NY 14502
phone 315-986-8090
fax 315-986-8091
Item ID = F41
F57 bag material - 1 square yd $285.00 plus shipping

F. Sample preparation

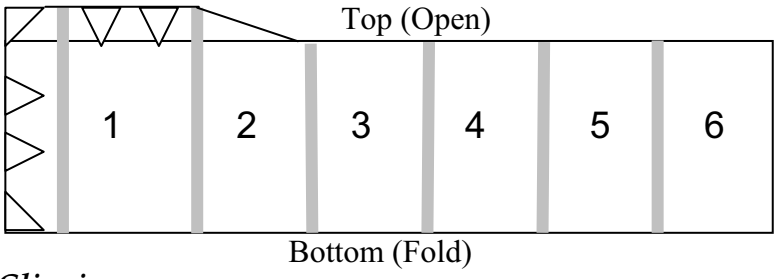
1. Grinding

All samples should be ground as per EP-04 to increase surface area and speed time. The finer the material is ground, the quicker the extraction process proceeds. The Wig-L-Bug is particularly effective for tree ring samples and has less sample loss than the Wiley Mill.

NOTE: Trials were run on intact tree-ring discs, but subsequent grinding was impossible in the roller grinders, and the Wiley Mill left a fluffy – cotton-like pulp easily lost to the mill and difficult to condense into a weigh tin for isotopic analyses.

2. Extraction Bag Preparation

- cut ANKOM filter paper in 16 x 9 cm rectangles (the small plastic paper cutter works well for this).
- fold lengthwise.
- make sure, that **shiny** side of filter paper is on the **inside** of the bag for easier removal of the cellulose.
- leave 1 cm at the left side and seal to form six equal pouches (see figure, shaded lines represent seals) with the ANKOM heat sealer (heater setting 4). Do not seal top! Trim top as shown in the figure, but do not cut marks yet.



3. Bag Clipping

See Appendix for examples and Cellulose Submission IDsheets. Each packet of six sample bags will have a unique clipping to identify it to the samples on the IDsheet. The picture of the clip

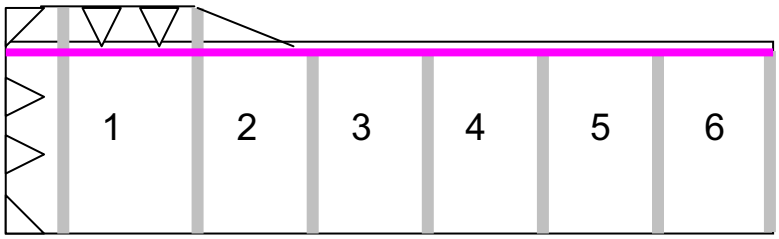
code for a packet is located next to the location for data entry on the IDsheet. There are three IDsheets, designating three different groups of samples A, B and C for the three different soxhlets. Each group contains 8 packets (A-H) for a total of 48 samples in each group to be run together in a particular soxhlet. If the number of samples is less than 144, then decrease the number of packets in a group. That way each soxhlet will contain the same number of samples.

- Once the number of packets has been determine, prepare all the empty packets and clip the codes as indicated on the IDsheets.
- If there are fewer than 8 packets per group, cross out the data section on the IDSheet for those packets that will not be used (i.e. if there are 7 packets, cross out A-H, B-H, and C-H).
- Place empty clipped packets in order on the appropriate sample IDSheet.

All bag groups are numbered in the same way. The 1st sample is placed in the pocket closest to the identification markers. The next numbers (2 through 6) are placed in the next pocket down the line in order (See above figure)

4. Sample loading and submission

- Fill out your name, date and project on the top of each IDSheet.
- Beginning with Group A, packet A, take the appropriate clipped packet (**Double check pattern on IDsheet**) and the first six sample vials containing the plant material to be processes.
- Place the vials in order 1-6, and fill in the sample ID data from the vial into the corresponding location on the IDsheet. **Double check the sample order (1-6) and the packet ID with the datasheet ID where the data has been recorded**.
- Place the plant material into the appropriate pocket on the packet starting with 1 (see figure). Be sure to get all the material into the pocket and to not get any into the other pockets. Tap to get the material into the bottom.
- Once all six pockets are full in a packet, seal the top of the packet which will seal each pocket closed (Pink line in figure). Double check that each pocket is sealed.
- Place empty sample vials back in order because they can be used again once material is processed.
- Repeat filling packets in order on the IDSheet until all packets are full



- Copy the final IDSheets and keep originals in your records, and keep the copies in the processing room for sample reference.

G. Extraction of waxes, oils and resins

WHOLE PROCEDURE SHOULD BE CARRIED OUT UNDER A FUME HOOD

The main difficulties are:

Water leakage flooding the room (e.g. clogged sink, broken lines), and
Boiling the samples dry (make sure connections are tight and condensers are cool).

1. ***Soxhlet extraction using toluene and ethanol***
   - Reaction time: ~ 44 hrs / over two nights.
   - place up to 50 samples in the Soxhlet apparatus.
   - fill round, flat bottom flask with (Carefully, materials are harmful!):
     6 – 8 boiling chips (use mirror to check if you're not sure they're there)
     250 ml toluene and
     125 ml ethanol (equals 2:1 toluene/ethanol mixture)
   - place flask in heating mantle and assemble rest of Soxhlet apparatus.
   - attach rubber hoses to water outlet, condenser and sink to supply cooling water (without cooling, mixture evaporates out through condenser and hood and flask burns!) to condenser, SECURE HOSES.
   - heat toluene/ethanol mixture to boiling point and adjust heat to steady boil (good heat settings is 8).
   - uneven boil is due to lack of boiling chips. Mirror can be used to verify whether the chips are present.
   - adjust flow of cooling water (flow has to be quite strong – 3rd *condenser should not be warm*!).
   - after reaction is complete (~44 hours), turn off heat and unplug, leave cooling water running until apparatus has cooled down to minimize evaporation (approx. 20 minutes).
   - then, turn off water and carefully take Soxhlet apparatus apart and dispose of Toluene-Ethanol-Mixture in labeled waste jug, retrieve boiling chips (dispose of them separately – allow to evaporate in hood, then throw in trash).

2. ***Soxhlet extraction using 100 % ethanol***
   - Rinse 3 times with 75 ml Ethanol, leaving samples in extractor.

   - Repeat steps listed under a) with 400 ml 100% ethanol and no tolulene (make sure to add new boiling chips!).

   - After decanting the ethanol to a labeled waste jug, fill the soxhlet to the neck with RO water and let flush through to the sink several times. Repeat this step 5 – 6 time until the flushed solution runs clear.

   - Then pour samples from each soxhlet into the boiling water for the next step – a microwave will greatly speed the time to boil the water.

H. Boiling – Water Soluble Extraction

CARRIED OUT UNDER A HOOD

1. ***Boiling***
   You've switched to water now – make sure not to boil dry!!!
   - Reaction time: 6 hrs.
   - place stir-bar and dried samples in 1000 ml beaker on heated stir plate.
   - add ~700 ml boiling RO water (the microwave heats the water much faster!), and turn on the stir mechanism.
     NOTE: Use a 500 ml Ehlermyer flask floating on the surface as lid to keep samples submerged. ***Fasten flask the neck loosely to a ring stand so the flask will move with water level.***

Reasonable heat and stir setting to maintain stirring action and boiling water for the particular heated stir-plates labeled as set 0, 1, and 2 are:

	Set 0	Set 1	Set 2
Stir setting =	slowest setting is fine		
Heat Setting =	6.8	6.2	6.0

- add more RO water about every 1.5 to 2 hours for 6 hours (microwave the water first, so the boiling does not stop).
- turn off heat and decant water into sink.

2. *Rinsing*

Important for complete removal of waxes and resins from solution.

- add enough RO water to cover the samples (use flask lid to keep samples submerged.
- place on stir-plate and stir lightly for 20 - 30 minutes.
- decant solution into sink.
- repeat rinsing process 6 - 7 times.
- leave samples in water over the weekend (or air dry if break before bleaching step).

I. **Bleaching - Lignin Extraction to Produce Holocellulose**

ALL PROCEDURES CARRIED OUT UNDER THE HOOD

NEVER REMOVE ANY FLASK CONTAINING SODIUM CHLORITE AND GLACIAL ACETIC ACID FROM THE HOOD

Notes:

- **Make sure that the solution is bright yellow, has a pH < 4.05, a temperature of 70 - 75°C and that the samples are floating.**

- Sodium Chlorite is the actual bleaching agent and only reacts under acidic conditions and high temperature. To reduce hazards, add sodium chlorite before adding the acetic acid.
- During the reaction, Cl is used up and the pH is likely to rise, which would stop the reaction. Therefore, measure pH and temperature regularly. pH-measurement is only possible with an electronic ph-Meter. Any pH paper will be bleached and unusable due to the sodium chlorite.
- Sufficient sodium chlorite is present when the solution is yellow. Add more if the solution is brown, clear, or light yellow.
- Sodium chlorite raises the pH, so glacial acetic acid should be added in proportion to keep the pH below 4.0 (e.g. 1ml acid generally lowers the pH by 0.1pH).
- The reaction is over when the solution remains yellow and the pH does not increase over a 12 hour period.

1. *Bleaching*

- Reaction time: 2 - 5 days.
- Bleaching solution:
  - add 7-8 g of sodium chlorite to 0.8 liters of water in 3 Erlenmeyer flasks
  - place on heated stir-plate and turn on stir mechanism. Slowest stir setting is fine, stir until well mixed.
  - Add 4 ml of acetic acid to each flask and heat as follows:

	Set 0	Set 1	Set 2
Heat Setting	4.5	2.4	2.4

- Heat solution to 70 -75 °C

- place stir-bar and samples in 1000 ml beaker and place on hot plates (same heat settings as above.
- Add Bleaching solution to beakers (~800 ml per beaker)
- carefully monitor temperature and keep it steady between 70 - 75°C
- (if heated to 200°C toxic Cl and Na_2O fumes develop!!). Tilt the flask-lid to side to submerge hand-held thermometer (glass or electronic) into solution.
- once the solution has reached temperature, measure the pH by inserting the electronic pH probe into the solution as above. The pH meter must be calibrated in pH 7.0 and pH 4.0 buffers at the start of each day (write these calibrations in the comments section of the Bleach Monitoring data sheet – see attached). We found that calibrations at room temperature (26 degrees C) held when the temperature pH meter setting was adjusted to 70 degrees C and the buffers were also heated to 70 degrees C. Therefore, calibrations at room temperature should suffice for measurements collected at warmer temperatures so long as the pH meter is set to the same temperature as the solution.
- check the temperature and pH at the start, mid and end of each day and fill in all columns on the Bleach Monitoring data sheet.
- if pH rises during the first day add 1 – 2 more ml of the glacial acetic acid to reduce the pH to 4.0 and keep the reaction going.
- Sodium Chlorite should be added at the start and end of the first day, and any time the solution loses its bright yellow color or the pH raises by more than 0.1 thereafter (generally no more than once per day after the first day). Acid should also be added with the sodium chlorite to maintain the pH at 4.0.
- also add boiling water when levels get low (Generally needed at the start and end of each day – addition of cool water slows reaction and alters pH!!!).
- continue reactions until the solution remains yellow and the pH does not increase over a 12 hour period.
- However, reactions must stop and samples rebagged after 5 days even if samples are not completely bleached because the bags will disintegrate!!!
- decant the solutions from all beakers into a common waste container and neutralize to pH 7 with ~6 molar NaOH (44g NaOH /1 liter water) before pouring it down the sink (note: if you are making α-cellulose you will have NaOH waste solution which can be used for this).
  - **Make sure** to alter the temperature of the pH meter to match that of the decanted solution so the pH reading is correct.
  - **Also - remove the stir bar** and press a smaller flask or beaker against the samples to squeeze out remaining solution – this speeds the process considerably!

2. *Rinsing*

- add enough RO water to cover the samples (about 400 ml) and place back on the stir-plate for about 20-30 min.
- repeat rinse step 4-5 or more times.
- For the final rinse of the extraction (note rinsing occurs 3 separate times for α-cellulose) decant the solution from each beaker separately (including pressing the solution from the sample bags) until the conductivity is <10 μS/cm as measured with an electronic conductivity meter.
  - Check the calibration of this meter with a 1.990 mS/cm solution (1000mg NaCl/1 liter water) and RO water (should be less than 10 μS/cm).

- Make sure to flick sensor dry between solutions, and repeat submission in the same solution twice before taking a reading.

3. a-Cellulose (removal of holocellulose) only necessary for $\delta^{18}O$

- NaOH Solution: Add 170 g of NaOH to 1 liter of water in an Erlenmeyer flask, stir rapidly. Add no heat! The flask will get hot since the dissolving NaOH is exergonic.
- Add 500 mls or so to beakers with rinsed samples, stir for 45-60 min. NO HEAT.
- decant the solutions from all beakers into a common waste container.
- Follow rinsing procedures about for about 3-5 rinses (don't worry about conductivity readings).
- Acetic Acid solution – Add 100 ml acetic acid into Erlenmeyer flask and add RO water to make 1 liter, stir.
- Add 500 mls of solution into each beaker and stir for 45-60 min, no heat.
- Decant solution into NaOH common waste to neutralize both, check for pH of 7 before disposing.
- Follow rinsing procedures above (check conductivity), may leave in rinse overnight if needed.

4. Bag Removal

- Remove samples from flasks (leave stir-bar) and dry samples in oven at 70 °C over night. Keep the different groups in order.
- **Place the packets in order with the appropriate IDSheet.**
- Get the empty sample vials and be sure that order is also correct. Clean vials with lab air if necessary (we found it was easy to clean vials with the vacuum sink spout in the north sink).
- Starting with Packet A-A, get the six appropriate sample vials for that packet and set in order. **Double check vial labels with IDSheet information for that packet.**
- Open one sample pocket at a time starting with pocket 1 and carefully transfer the cellulose to the appropriately labeled vial.
- Repeat until all the packets are empty, and samples are safely closed in appropriate labeled vials.

J. Preventive Maintenance and Corrective Action

1. Soxhlet Extraction Precautions

The soxhlet extraction part is the most important step of the whole procedure. If the samples are not completely freed of waxes and resins the bleaching will not be successful. It therefore is very important to rinse several times after completing the extraction. Samples with high resin content and samples with low resin content should be run in separate batches. Wood samples should also be run separately from leaf or needle samples. For samples with low resin content the mentioned extraction times are sufficient. If the samples contain relatively high resin amounts or the resin amount is unknown, extraction times should be extended up to 48 h per extraction.
A strong flow of cooling water is necessary to minimize evaporative loss during the soxhlet extractions. A small loss however can not be avoided. This loss normally does not affect the procedure but should the liquid level get too low ethanol or toluene-ethanol-mixture can be added at any time. It is also important to ensure the strength of the hoses and that the sink will not get clogged – such that the water continues to exit the drain and the lab does not become flooded.

2. Reduced Extraction Bag Integrity with Bleaching

The bleaching will take about four days for wood samples, but might take considerably longer for leafy material due to the high chlorophyll content. After four to five days however the samples should be rinsed and dried regardless of the completion of the bleaching process. This is necessary because the sample bag material weakens during the bleaching due to the effects of the acetic acid-sodium chlorite mixture. If samples are treated for more than five (maybe six) days without replacement of the bags, there will be an increasing risk that the bags will disintegrate or that the seals will break

a.

K. Quality Assurance / Quality Control

Samples that have gone through the above steps should turn out purely white with only holocellulose remaining. Since extraction lengths vary with tissue matrix, we have doubled the length of the soxhlet extractions over that published by Leavitt and Danzer (1993). However, remnant waxes and resins would prevent completion of the bleaching process to a white material, and lignins are thoroughly removed when the sodium chlorite remains in the solution and the pH is maintained below 4.05 for a 12 hour period. Leuenberger et al.(1998) have shown that samples run through cellulose extraction processes twice do not vary in isotopic composition (see attached).

L. References

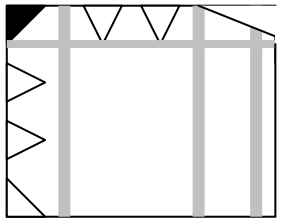
Leavitt, S. W., and S. R. Danzer. 1993. Methods for batch processing small wood samples to holocellulose for stable-carbon isotope analysis. Analytical Chemistry **65**:87-89.

Leuenberger, M., S. Borella, T. Stocker, M. Saurer, R. Siegwolf, F. Schweingruber, and R. Matyssek. 1998. Stable isotopes in tree rings as climate and stress indicators. vdf Hochschulverlag AG, Zurich.

Sternberg, L. 1989. Oxygen and hydrogen isotope measurements in plant cellulose analysis. Pages 89-99 *in* H. F. Linskens and J. F. Jackson, editors. Modern Methods of Plant Analysis Vol 10: Plant Fibers. Springer Verlag, Berlin.

Name _____ Date_____ Project_____

M. Appendix 1: Cellulose Submission IDSheets



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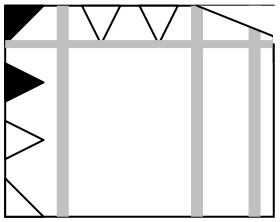

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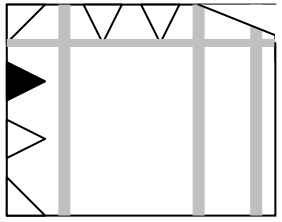

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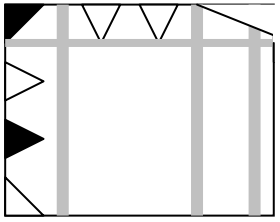

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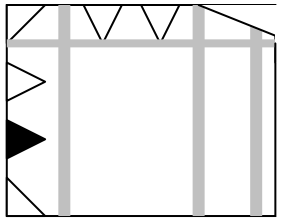

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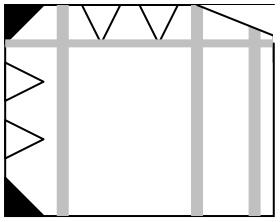

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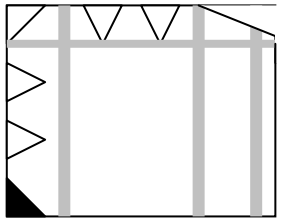

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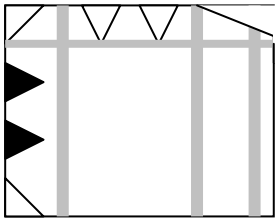

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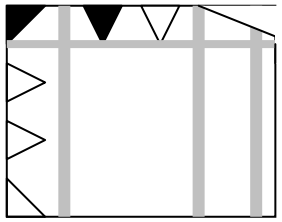

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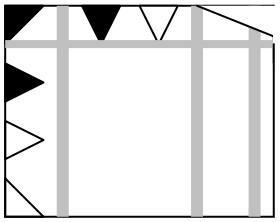

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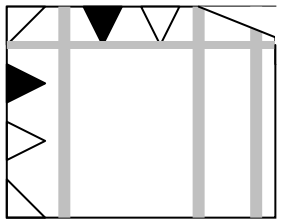

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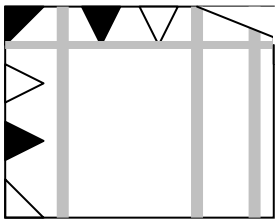

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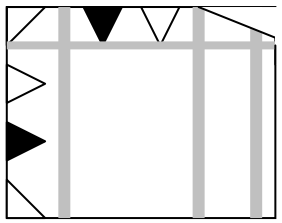

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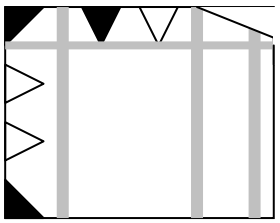

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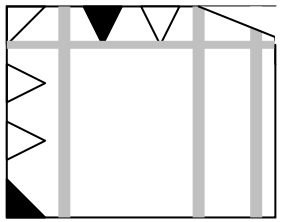
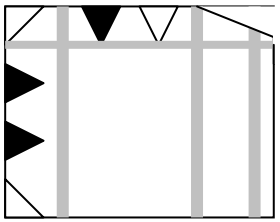


Group ID	#	Sample code	comments
B D	1		
	2		
	3		
	4		
	5		
	6		
Group		Sample	



Group ID	#	Sample code	comments
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	3		
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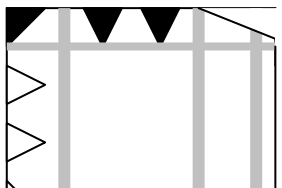
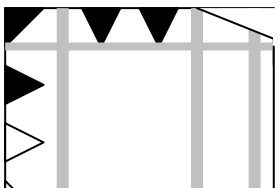
	5		
	6		
Group		Sample	





Name _____ Date_____

Project_____

ID	#	code	comments
C A	1		
	2		
	3		
	4		
	5		
	6		

ID	#	code	comments
C E	1		
	2		
	3		
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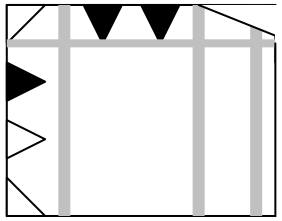
	5		
	6		



Group ID	#	Sample code	comments
C B	1		
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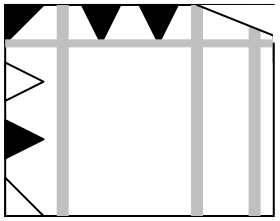

Group ID	#	Sample code	comments
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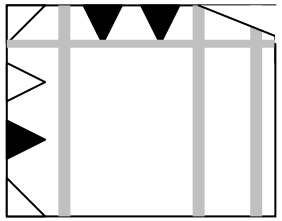

Group ID	#	Sample code	comments
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	3		
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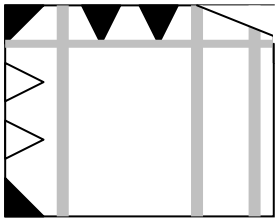

Group ID	#	Sample code	comments
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	3		
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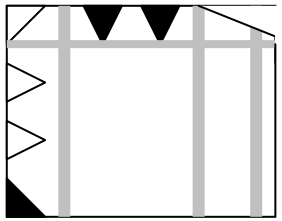

Group ID	#	Sample code	comments
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	2		
	3		
	4		
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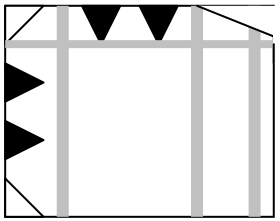

Group ID	#	Sample code	comments
C H	1		
	2		
	3		
	4		
	5		
	6		